Cloning, expression, purification, and characterization of zebrafish cytosolic serine hydroxymethyltransferase

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Abstract

A cDNA which encodes for zebrafish serine hydroxymethyltransferase (SHMT) has been cloned into a pET43.1a vector as a NdeI–EcoRI insert and transformed into HMS174(DE3) cells. After induction with isopropyl thiogalactoside, the enzyme was purified with a three-step purification protocol and about 15 mg of pure enzyme was obtained per liter of culture. Spectral and structural characteristics of the recombinant zebrafish SHMT are similar to the rabbit and human cytosolic SHMT. Kinetic constants for the natural substrates L-serine and tetrahydrofolate are also comparable to the values obtained previously for the rabbit and human cytosolic enzyme.

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Serine hydroxymethyltransferase (SHMT, EC 2.1.2.1) reversibly converts serine and $\text{H}_4\text{PteGlu}$ to glycine and 5,10-CH$_2$-$\text{H}_4\text{PteGlu}$, the principal pathway for incorporation of one-carbon units in the biosynthesis of numerous cell metabolites. Found in high levels in rapidly proliferating cells compared with resting cells, this enzyme is suggested to be a potential target for chemotherapy [1–3]. SHMT is present in virtually all cell types and has been widely studied in many living systems including prokaryotes, vertebrates, mammals, and plants [4]. In higher organisms more than one SHMT isoforms is often present: a cytosolic isoform and an organelle-associated form, usually mitochondria. SHMT also catalyzes the conversion of 5,10-CH$_1$-$\text{H}_4\text{PteGlu}$ to 5-CHO-$\text{H}_2\text{PteGlu}$, which has been proposed to play a role in the regulation of folate metabolism [5,6]. Nearly all of the studied prokaryotic SHMTs exist as homo-dimers, whereas homo-tetramers are the major form found in eukaryotes [7].

The cDNA for SHMT has been cloned from numerous sources. These cloned SHMTs have been purified and their functions and structures extensively studied in many living systems including Escherichia coli, rabbit, mouse, and human, but not zebrafish, a vertebrate that has risen to be a prominent animal model. The features of external development, transparent embryo, ease of growth and breeding, and economy have made zebrafish an ideal animal model in the laboratory. Despite being distant from mammals, zebrafish nevertheless has comparable organs and tissues, such as heart, kidney, pancreas, bones, and cartilage. The similarities and shared features between zebrafish and mammalian biology have led to the development of many zebrafish pathogenesis models and disease-related assays for drug screening and human diseases, including those related to: organ or tissue development; angiogenesis; hemostasis; heart function and circulation; apoptosis and proliferation; lipid metabolism; inflammation; drug abuse and addiction; and toxicology and teratogenicity [8]. However, little has been done about folate and folate-mediated one-carbon metabolism in the zebrafish, even though it has
been known that this metabolic pathway plays a significant role in fetal development, especially in the neural tube and heart in mammals, as well as in oncogenesis [9,10].

In the current study, we clone the complete encoding sequence of zebrafish SHMT, (zSHMT) into an expression vector and purify the overexpressed enzyme. The properties and functions of zSHMT, including stability, catalytic activity and kinetics, and coenzyme binding stoichiometry are reported.

Materials and methods

Materials

PCR primers were ordered from MDBio (Taiwan). The SMART RACE amplification kit was purchased from Clontech (California, USA). PCR Master Mix was purchased from ABgene House (Surrey, UK). Enzymes used for cloning procedures were purchased from either BRL or New England BioLabs (Maryland, USA). The clone expressing 5,10-methylenetetrahydrofolate dehydrogenase was a generous gift from Dr. Verne Schirch/Virginia Commonwealth University, Richmond, VA, USA. The HPLC gel filtration column Alltech ProSphere SEC, 250 HR, S-200 (4.6 mm × 30.0 cm) was purchased from Alltech (Illinois, USA). (6S)-Tetrahydrofolate monoglutamate was a generous gift from Dr. Doris Vrontakis, Switzerland. All other chemicals, including coenzymes, buffers, amino acids, and antibiotics were purchased from Sigma–Aldrich Chemical (Montana, USA).

Fish care and maintenance

Zebrafish (Danio rerio, AB strain) were bred and maintained in a 14–10 h light–dark diurnal cycle according to the standard condition described by Westerfield [11]. Embryos were staged according to Kimmel et al. [12].

Preparation of cDNA library from zebrafish embryo

Total RNA from zebrafish embryos was isolated by the single-step method using RNAzol B reagent (Tel-Test, Inc.) [13]. Briefly, 200 zebrafish embryos at the stage of 24-h post-fertilization were homogenized with 5–20 strokes in RNAzol B reagent with a sterilized plastic mini-mortar. Chloroform/isooctyl alcohol (24:1) of one-tenth volume of homogenate was added and followed by vigorous shaking. After a brief incubation on ice, the mixture was centrifuged and the upper aqueous phase transferred to a clean centrifuge tube. An equal volume of isopropanol was added for precipitating RNA. After a 15-min incubation at 4 °C, the RNA precipitate was collected by centrifugation followed by washing twice with 75% ethanol and dried under vacuum.

The dried RNA was redissolved in DEPC-treated water and subjected to poly(A)+ RNA isolation using a Dynabead mRNA Purification Kit (Dynal Biotech, Inc.). In brief, an equal volume of binding buffer (20 mM Tris-HCl, pH 7.5, 1.0 M LiCl, and 2 mM EDTA) and total RNA were combined and heated to 65 °C to disrupt RNA secondary structure. After cooling on ice, binding buffer-equilibrated magnetic beads containing oligo(dT)25 was added into the above RNA solution, incubated at room temperature for 5 min with gentle rotating and then washed twice with buffer B (10 mM Tris–HCl, pH 7.5, 0.15 M LiCl, 1 mM EDTA). Elution of poly(A)+ RNA was initiated by adding 100 µl of elution buffer containing 10 mM Tris–HCl, pH 7.5, into the beads and heated at 65–80 °C for 5 min. Eluted poly(A)+ RNA was stored at −70 °C or used immediately in the subsequent cDNA construction reaction.

The zebrafish embryo cDNA library was constructed using the SMART-RACE cDNA Amplification Kit (Clonetech). Two separate reactions were carried out to set up 5′-RACE and 3′-RACE cDNA. Briefly, for 5′-RACE cDNA, poly(A)+ RNA, SMART II oligo and 5′-CDS primer were combined and heated to 70 °C. This mixture was then cooled to room temperature for annealing of the primer to poly(A)+ RNA. Reverse transcription was initiated by adding buffer, a dNTP mixture and MMLV reverse transcriptase to the annealing mixture and reaction was performed at 42 °C for 1.5 h. 3′-RACE cDNA was constructed in almost the same way, except that only poly(A)+ RNA and 3′-CDS primer was combined at the initial step. The resultant cDNA mixture was diluted with Tricine–EDTA buffer and stored at −70 °C in aliquots.

Bacterial strains and plasmids

The E. coli strain HMS174(DE3) [F− recA rcl 12 m 712 Rif], which contains the T7 RNA polymerase gene was used both for the construction of clones and for protein expression. The pET43.1a plasmid and E. coli HMS174 (DE3) strain were obtained from Novagen.

General cloning procedures

Digestion with restriction enzymes, PCR polymerization, ligations, and dephosphorylation with calf intestinal alkaline phosphatase were carried out according to the manufacturer’s instructions (New England BioLabs, Inc.). Plasmid DNA purification and DNA extraction from agarose gels were performed using spin purification products (Viogene, Taiwan). Transformations by calcium chloride, growth of cells, and electrophoresis were performed by standard procedures as described by Sambrook et al. [14]. Competent cells for transformation were made by the standard calcium chloride method. All PCR experiments were carried out in a total volume of 25 µl. PCR contained 10 ng 5′-RACE cDNA, 5 pmol of each primer and 1× Extensor Hi-Fidelity PCR Master Mix containing 2.25 mM MgCl2, 1.4 mM dNTP, 20 mM Tris–HCl, and 1.25 U of total DNA polymerase. Reactions were carried out in 0.2 ml PCR tubes. Amplifications were performed
in a Thermo Hybaid/thermocycler (UK) using the following reaction conditions: 1 min at 94 °C; 40 cycles of 30 s at 94 °C, 30 s at 60 °C, and 90 s at 68 °C. After 40 cycles, the reaction was continued at 68 °C for 5 min and then brought to 4 °C. The PCR solutions were then analyzed by agarose gel electrophoresis.

Cloning strategy

A pair of primers were designed based on the zebrafish SHMT-1 (zSHMT-1) cDNA available in GenBank (GenBank Accession Nos. BC055527 and BC066496) to PCR amplify the complete SHMT encoding sequence from zebrafish 5'-RACE-Ready cDNA library. A fragment of 1.5 kb was obtained using the following primers: 5'-gctagctctatatttccacgaagttcatcacatatg (forward) and 5'-ggacagagaattctcccagcgcaacggtcatac-3' (reverse). To simplify the cloning procedure, two restriction enzyme sites: NdeI and EcoRI (underlined), were introduced into the designed forward and reverse primers, respectively. The PCR amplified product was first TA-cloned into pGEM-T plasmid (Promega, WI, USA) and subcloned into the expression vector pET43.1a between NdeI and EcoRI sites (Fig. 1). Successful cloning of the complete zSHMT-1 was confirmed by both restriction enzyme digestion and DNA sequencing. Fig. 1 shows a map of plasmid pET43.1a::zSHMT-1. The resultant constructs were transformed into an E. coli host cell HMS174(DE3) for enzyme expression and purification.

Expression and purification of recombinant zSHMT-1

All buffers described below for the purification of zSHMT-1 contained 5 mM of 2-mercaptoethanol, 0.2 mM PLP unless otherwise stated.

Escherichia coli containing the desired plasmid was grown overnight at 37 °C in 200 ml of Luria broth with 100 μg/ml ampicillin. This culture was then used to inoculate three flasks, each containing 1 liter of YT broth plus 75 μg/ml of ampicillin. The inoculum was grown at 37 °C with vigorous shaking until the OD600 reached 1.0, then IPTG was added to a final concentration of 0.08 mM, and the cells incubated for another 3 h at 25 °C with vigorous shaking. Cells were harvested by centrifugation and the cell pellet resuspended in 100 ml of 50 mM Tris-HCl, pH 8.0, containing 2 mM EDTA. Lysozyme, 10 mg dissolved in 5 ml of the same buffer, was added to the cell suspension and then incubated for 15 min at room temperature before freezing overnight at −80 °C.

The lysed cells were thawed and resuspended in 200 ml of 20 mM potassium phosphate, pH 7.0. Protamine sulfate, 200 mg dissolved in 5 ml of 20 mM potassium phosphate, pH 7.0, was added to the lysis suspension with constant stirring. A white precipitate formed which was removed by centrifugation for 15 min at 13,000 g. The pellet was discarded and a 55–75% ammonium sulfate fractionation was performed. The 75% saturation of ammonium sulfate precipitation pellet was resuspended in 10 ml phosphate buffer and dialyzed overnight at 4 °C in 2-liters phosphate buffer, pH 7.0.

The desalted protein solution was loaded onto a CM-Sepharose column (3 × 12 cm) equilibrated with 20 mM potassium phosphate, pH 7.0. The column was washed thoroughly with equilibration buffer until the A280 was less than 0.1. The zSHMT-1 was eluted from the column as a yellow band with a linear gradient of 150 ml of equilibration buffer and 150 ml of 300 mM potassium phosphate, pH 7.0. Fractions containing activity were pooled, and the protein solution was concentrated by precipitation with 75% ammonium sulfate. After centrifugation the redissolved pellet was dialyzed overnight against 20 mM potassium phosphate, pH 7.2, containing both 2-mercaptoethanol and EDTA as above. Pyridoxal-5'-phosphate was also added to the dialysis buffer to 0.1 mM.

The protein solution from above was loaded onto a hydroxyapatite column (2.5 × 5 cm) equilibrated with 20 mM potassium phosphate, pH 7.2. The column was washed with equilibration buffer until the A280 was less than 0.1, and the enzyme was eluted with a linear gradient of 100 ml of equilibration buffer and 100 ml of 300 mM potassium phosphate, pH 7.2. Fractions containing activity were pooled, and the enzyme was concentrated by ammonium sulfate precipitation. The pellet was dissolved in 20 mM potassium phosphate, pH 7.0, containing 0.1 mM PLP and was dialyzed overnight in the same buffer. To the protein solution was added 10% glycerol before storing at 4 or −80 °C for longer storage. Protein from each step of the purification protocol was analyzed by SDS-PAGE under reducing conditions on a 10% gel.

Enzyme assays

SHMT catalyzes the cleavage of serine and H4PteGlu to form glycine and 5,10-CH2-H4PteGlu. The rate of 5,10-CH2-H4PteGlu formation can be continuously

Fig. 1. Map of plasmid pET43.1a::zSHMT-1 used to express zSHMT in HMS174(DE3) cells. The construction of this plasmid is described under Materials and methods.
monitored at 340 nm by coupling with excess 5,10-CH₂-THF dehydrogenase, which converts NADP⁺ to NADPH [15]. The extinction coefficient of 7200 M⁻¹ cm⁻¹ at 340 nm was used to measure the generation of NADPH, which corresponds to the production of 5,10-CH₂-H₄PteGlu. An assay contained, in 1 ml, 20 mM potassium phosphate, pH 7.0; 5 mM of 2-mercaptopethanol; 100 mM NADP⁺; 100 μM H₄PteGlu; 20 mM l-serine; 5 μg of 5,10-CH₂-H₄PteGlu dehydrogenase; 1–10 μg protein from the E. coli expressed zSHMT-1 extract. An enzyme unit is defined as the formation of 1 μmol of product/min at 30 °C. Assays and protein determinations were done with a Helios spectrophotometer.

**Determination of kinetic constants**

Apparent Kₘ and kₗₑₚ values for l-serine and H₄PteGlu were determined with the coupled enzyme assay which determines the initial rate of formation of the product 5,10-CH₂-H₄PteGlu as previously described [15]. l-Serine concentrations were varied from 0.07 to 0.75 mM. The kinetic constants were determined from double-reciprocal plots of initial velocity versus substrate concentration. All reactions were performed at 30 °C in 20 mM potassium phosphate containing 5 mM of 2-mercaptopethanol, pH 7.0.

**Determination of dissociation constant for H₄PteGlu**

The dissociation constant for H₄PteGlu of zSHMT-1 was measured by the formation of a ternary enzyme–glycine–H₄PteGlu complex absorbing at 495 nm at various concentrations of H₄PteGlu as described previously [16]. Increasing concentrations of H₄PteGlu, ranging from 2.5 to 50 μM, was added in a 1 cm cuvette in the presence of 10 μM ZSHMT and 100 mM glycine. Results of the absorbance at 495 nm and H₄PteGlu concentration were analyzed with both double-reciprocal and Scatchard plots yielding the zKₐ and stoichiometry of bound H₄PteGlu.

**Preparation of zSHMT-1 apoenzyme**

Zebrafish SHMT-1 contained tightly bound PLP. This was removed to prepare the apo enzymes by chromatography on a 0.7 × 4-cm phenyl-Sepharose column equilibrated with 30% ammonium sulfate in 50 mM potassium phosphate, pH 7.2, and containing 10 mM l-cysteine. Under these high salt conditions the enzyme binds to the phenyl-Sepharose. The bound PLP reacts with l-cysteine to form a thiazolidine complex, which has reduced affinity for the enzyme and dissociates to leave column bound apo-zSHMT-1. After removing all of the PLP with the equilibration buffer, apo-zSHMT-1 was eluted with 20 mM potassium phosphate, pH 7.2, containing 2-mercaptopethanol.

**Determination of size of apo- and holo-zSHMT-1**

The polymeric property of both apo- and holo-zSHMT-1s was examined by size-exclusion chromatography. Enzymes were chromatographed on a Superdex 200 size exclusion column (0.46 × 30.0 cm) equilibrated with 20 mM potassium phosphate, pH 7.0, containing 100 mM NaCl and 5 mM of 2-mercaptoethanol on an Agilent 1100 HPLC. The retention volume was compared to the following standards: apo-ferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), carbonic anhydrase (29 kDa), and ribonuclease A (14 kDa).

**Determination of molar extinction coefficient**

The predicted molar extinction coefficient for zSHMT-1 was determined from its amino acid composition via the computer program, Vector NTI Suite 8, based on the method of Gill and von Hippel [17]. The actual coefficient was determined by multiplying the predicted value by the ratio of absorbance at 278 nm in 8 M urea (denatured state) and 20 mM potassium phosphate, pH 7.25 (native state), for a dilute solution of the respective zSHMT-1.

**Determination of stoichiometry of PLP binding**

The amount of PLP bound to the zSHMT-1 was determined by the method of Ulevitch and Kallen [18] with minor modification. In brief, 5 mg of the holo-zSHMT-1 was extensively dialyzed against 20 mM potassium phosphate buffer, pH 7.3, containing 1 mM EDTA. Then following a 1-h equilibration of the enzyme with l-cysteine by adding l-cysteine to final concentration of 100 mM in centrifuge tubes, a 2-min immersion in boiling water and rapid cooling on ice, the denatured protein was removed by 15-min centrifugation. The number of moles of PLP was determined from the absorbance at 330 nm of the thiazolidine present in the supernatant.

**Results and discussion**

Serine hydroxymethyltransferase is a ubiquitous and highly conserved PLP-dependent enzyme that catalyzes the interconversion of serine and H₄PteGluₙ to glycine and 5,10-CH₂-H₄PteGluₙ. It holds the key in regulation of folate-mediated one-carbon metabolism. Impairment of SHMT activity might result in disturbance of intracellular one-carbon pool homeostasis and lead to pathogenesis, such as, homocysteinemia, cancers, cardiovascular diseases, and neural tube defects [19–22]. Moreover, SHMT, along with thymidylate synthase and dihydrofolate reductase, constitute the thymidylate cycle. Dihydrofolate reductase and thymidylate synthase have been favorite targets for designing and developing inhibitors for use as anticancer drugs. Since the common problem of drug resistance is often encountered in clinical situations during use of such inhibitors, there is a need to identify additional targets for cancer chemotherapy. SHMT, the key enzyme in one-carbon metabolism, has been suggested to be a target for chemotherapy [7]. Fully understanding this enzyme is therefore a necessity for employing zebrafish, an ideal
animal model for drug screening and as a tool for diseases studies and drug discovery related to folate-mediated one-carbon metabolism. Obviously, the prerequisite is to obtain large quantity of pure zebrafish SHMT for a thorough investigation on the structure and function of this enzyme. The most convenient method is to purify zSHMT from a bacterial clone overexpressing this enzyme, since this creature is small and purifying proteins from their organs or tissues is not feasible.

**Sequence analysis of recombinant zSHMT-1**

The zSHMT-1 cDNA clone we obtained (AY850381) was subjected to sequencing and compared with the three known zebrafish SHMT sequences available in GenBank (BC055527 and BC066496). Four amino acid residues at positions 4, 119, 450, and 459 are found to vary among the three sequences (Fig. 2). Interestingly, the residues at these four positions in the clone we obtained are identical to either one of the two known sequences. We suspect that these four positions might represent possible polymorphisms of this gene in zebrafish.

**Expression and purification of zSHMT-1**

Induction for zSHMT-1 was performed at 25°C with 0.08 mM IPTG for 3 h. We found that the expression of zSHMT-1 under this condition reaches an acceptable level, but with minimum production of an insoluble form of zSHMT-1. Higher concentrations of IPTG, increased induction temperature and/or prolonged induction time were found to increase the ratio of insoluble and soluble zSHMT-1, although the amounts of induced enzyme in soluble and insoluble fractions of cell extract were both increased. The ratios between zSHMT-1 present in soluble fraction and inclusion bodies are roughly 1:1 and 1:2 at 25 and 37°C, respectively (data not shown). It suggests that slowing down the IPTG induction by lowering the induction temperature and the concentration of IPTG had prevented the overexpressed zSHMT-1 from forming inclusion body [23]. It is also probable that induction at temperatures higher than 28°C results in increased insoluble enzyme since zebrafish is normally cultivated at 28°C.

The purification procedure for zSHMT-1 is very similar to the documented protocols for rabbit and human SHMT [24,25]. We used protamine sulfate to remove the nucleic acids and to decrease possible interference in subsequent chromatography steps. After ammonium sulfate precipitation and dialysis to remove the salt, zSHMT-1 binds to CM-Sephadex and is eluted at high salt with a large purification factor, as predicted from the similarity to rabbit and human SHMT. This greatly simplifies the purification and permits removing the great bulk of unwanted protein since most E. coli proteins do not bind to cation exchange resins.
under these conditions. This includes *E. coli* SHMT separating the endogenous enzyme from our cloned SHMT [25]. The SHMT eluted from this column is better than 90% pure as determined by SDS–PAGE (Fig. 3). A minor band of less than 50 kDa is removed by further chromatography on a hydroxylapatite column. As observed for both rabbit and human cytosolic SHMT, zSHMT-1 also binds to phenyl-Sepharose tightly in the presence of 30% ammonium sulfate as a bright yellow band on the top of the column. However, the purification from phenyl-Sepharose is not significant (data not shown) and therefore was abandoned in our purification protocol.

Protein from each step of the purification was analyzed by SDS–PAGE under reducing conditions on a 10% gel. One microliter each of the extract and ammonium sulfate fractions was loaded onto the gel. For the CM-Sepharose step, a 2-μl aliquot was used and for the hydroxylapatite step a 2-μl aliquot of a 10-fold dilution was used. The enzyme was judged to be greater than 95% pure after the hydroxylapatite column. From 3 liters of cells about 42 mg of pure zSHMT-1 was obtained with an overall yield of 65% (Table 1). The purified recombinant zSHMT-1 can be stored at −20 or −80 °C in the presence of 10% glycerol for at least 6 months without significant change in catalytic activity.

**Spectral properties**

The spectral properties of the recombinant zSHMT-1 appear to be similar to those of the other mammalian SHMTs that have been purified [24,25]. In addition to the absorbance at 278 nm, zSHMT-1 also displays a distinct absorbance peak at 428 nm with a 278/428 nm ratio of 6.0 (data not shown). The 428-nm peak corresponds to the internal aldimine formed between PLP and an active site lysine residue. All other SHMTs that have been characterized give a distinct spectral change in the presence of glycine and H₄PteGlu due to the formation of a ternary complex which absorbs at 495 nm. This long wavelength absorbance is attributed to a glycine anion in resonance with the bound pyridoxal phosphate and has been used extensively to determine the binding constants of H₄PteGlu.

**Table 1**

Summary of recombinant zSHMT-1 purification

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lysate</td>
<td>38</td>
<td>971</td>
<td>361</td>
<td>0.37</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>8</td>
<td>128</td>
<td>331</td>
<td>2.59</td>
<td>92</td>
<td>7.0</td>
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<tr>
<td>CM-Sepharose</td>
<td>77</td>
<td>48</td>
<td>239</td>
<td>4.95</td>
<td>66</td>
<td>13.4</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>2</td>
<td>42</td>
<td>233</td>
<td>5.46</td>
<td>65</td>
<td>14.8</td>
</tr>
</tbody>
</table>
and glycine [4]. The zSHMT-1 exhibited the same spectral properties with glycine and H₄PteGlu.

**Oligomeric properties of zSHMT-1**

SDS-PAGE of recombinant zSHMT-1 showed a single band with an estimated size of 53 kDa (Fig. 3). This compares to the calculated size of 53,317 Da based on the amino acid sequence of this protein. The retention volume during chromatography on a Superdex 200 column showed that both holo- and apo-zSHMT-1 had a Stokes radius close to a globular protein of 200 kDa and were eluted at the same retention volume as rcSHMT tetramers (Fig. 4). The SDS-PAGE pattern and gel filtration elution profile of the enzyme suggest that zSHMT-1 is also a homo-tetramer as observed for rabbit and human SHMTs.

**Steady-state kinetic constants**

The recombinant zSHMT-1 catalyzes the retroaldol cleavage of serine in the presence of H₄PteGlu to form glycine and 5,10-CH₂-H₄PteGlu. An initial velocity study was done with L-serine serving as the variable substrate in the presence of saturated H₄PteGlu. Double-reciprocal plots of initial velocity versus serine concentration permitted the determination of both apparent $K_a$ for serine and $k_{cat}$ (Table 2). The $K_m$ value for serine is comparable to the values for rcSHMT and hcSHMT. The turnover number for zSHMT-1 is close to half of the rcSHMT and hcSHMT. The enzyme remains active and even has a higher turnover rate at 37 °C than at 30 and 28 °C (data not shown).

**Dissociation constants for H₄PteGlu**

As mentioned, the addition of H₄PteGlu, results in the loss of the α-proton of glycine in the active site and leads to formation of quinonoid complex which absorbs at 495 nm with the molar extinction coefficient of 40,000 M⁻¹ cm⁻¹. The absorbance shows saturation kinetics with H₄PteGlu, [26–28]. The binding of substrates to rabbit cytosolic SHMT is a sequential random mechanism. The clone we report here is possibly the cytosolic form of SHMT. As mentioned, the presence of subcellular SHMT isoforms is common in many organisms including humans, which has cytosolic and mitochondrial SHMTs. In fact, the partial peptide sequences of three possible isoforms of SHMT (deduced from the cloned cDNA fragments), including the zSHMT-1 reported in the present study, had been identified from the zebrafish 24-h embryo, first-strand cDNA library in our laboratory (data not shown). The complete peptide sequence of the zSHMT-1 shares about 80 and 63.0% identity with cytosolic and mitochondrial SHMTs, respectively, suggesting that the clone we report here is more likely a cytosolic isoform of zebrafish (Fig. 5). Meanwhile, lack of a leader peptide sequence also indicates the cytosolic localization of this enzyme. Currently, we have obtained another clone which possesses a putative leader peptide sequence and shares 84% identity with human mitochondrial SHMT.

**Table 2**

Comparison of kinetic parameters for recombinant zSHMT-1 with other forms of SHMT

<table>
<thead>
<tr>
<th>Species</th>
<th>$zK_m$ for serine (mM)</th>
<th>$zK_d$ for H₄PteGlu (µM)</th>
<th>Turnover number (min⁻¹)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zebrafish (zSHMT-1)</td>
<td>0.22</td>
<td>18</td>
<td>351</td>
<td>Present report</td>
</tr>
<tr>
<td>Human cytosolic</td>
<td>0.10</td>
<td>20</td>
<td>575</td>
<td>Kruschwitz et al. [24]</td>
</tr>
<tr>
<td>Rabbit cytosolic</td>
<td>0.30</td>
<td>20</td>
<td>630</td>
<td>di Salvo et al. [25]</td>
</tr>
<tr>
<td>Mouse</td>
<td>0.50</td>
<td>30</td>
<td>400</td>
<td>Strong et al. [22]</td>
</tr>
<tr>
<td>E. coli</td>
<td>0.30</td>
<td>25</td>
<td>640</td>
<td>Schirch et al. [33]</td>
</tr>
</tbody>
</table>
Further confirming experiments for determining the intracellular localization of this enzyme are underway.

**Conclusion**

In the present study, we clone, express, purify, and characterize SHMT from zebrafish. To our knowledge, this is the first example of a folate requiring enzyme from zebrafish that has been successfully and stably expressed in *E. coli*. Although a portion of the recombinant zSHMT-1 is present as inclusion bodies, it is possible to purify 15 mg of zSHMT-1 per liter of *E. coli* culture. The purification procedure for zSHMT-1 is similar to those for both human and rabbit cSHMTs, suggesting that these three enzymes are comparable in their properties and structures. It also suggests that the zebrafish is a suitable model for characterizing the physiological function of SHMT in vivo. The high purity of the recombinant enzyme will allow for antibody preparation and further structural characterization by X-ray crystallography, which is currently in progress. Steady-state kinetic studies and basic biochemical characterization of the recombinant zSHMT-1 also reveal similarity between this enzyme and human and rabbit cytosolic SHMTs. Moreover, the approximately 80% identity in amino acid sequence between zSHMT-1 and mammalian cytosolic SHMTs suggests that zSHMT-1 is also a cytosolic enzyme. Confirmation of the intracellular localization of zSHMT-1 and the cloning and
characterization of the other two prospective zebrafish SHMT isoforms are currently in progress.

Acknowledgments

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References

Serine Hydroxymethyltransferase Isoforms Are Differentially Inhibited by Leucovorin: Characterization and Comparison of Recombinant Zebrafish Serine Hydroxymethyltransferases

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ABSTRACT:

Serine hydroxymethyltransferase (SHMT) provides activated one-carbon units required for the biosynthesis of nucleotides, protein, and methyl group by converting serine and tetrahydrofolate to glycine and N5,N10-methylenetetrahydrofolate. It is postulated that SHMT activity is associated with the development of methotrexate resistance and the in vivo activity of SHMT is regulated by the binding of N5-CHO-THF, the rescue agent in high-dose methotrexate chemotherapy. The aim of this study is to advance our understanding of the folate-mediated one-carbon metabolism in zebrafish by characterizing zebrafish mitochondrial SHMT. The cDNA encoding zebrafish mitochondrial SHMT was cloned, overexpressed in Escherichia coli, and purified with a three-step purification protocol. Similarities in structural, physical, and kinetic properties were revealed between the recombinant zebrafish mitochondrial SHMT and its mammalian orthologs. Surprisingly, leucovorin significantly inhibits the aldol cleavage of serine catalyzed by zebrafish cytosolic SHMT but inhibits to a lesser extent the reaction catalyzed by the mitochondrial isozyme. This is, to our knowledge, the first report on zebrafish mitochondrial folate enzyme as well as the differential inhibition of leucovorin on these two SHMT isoforms. Western blot analysis revealed tissue-specific distribution with the highest enrichment present in liver for both cytosolic and mitochondrial SHMTs. Intracellular localization was confirmed by confocal microscopy for both mitochondrial and cytosolic SHMTs. Unexpectedly, the cytosolic isoform was observed in both nucleus and cytosol. Together with the previous report on zebrafish cytosolic SHMT, we suggest that zSHMTs can be used in in vitro assays for folate-related investigation and anti-folate drug discovery.

Folates carry the chemically activated single carbons at N5 and/or N10 positions and are required for the biosynthesis and metabolism of nucleic acid, amino acid, methyl group, neurotransmitter, and vitamins. Its vital role in nucleotide biosynthesis has led to the development of many anticancer drugs targeting folate-requiring enzymes. Among them, methotrexate (MTX) is one of the most widely used anticancer agents to date. It blocks de novo nucleotide synthesis by depleting reduced tetrahydrofolates mainly through inhibition of dihydrofolate reductase (DHFR) and thymidylate synthase (Fig. 1, enzymes 2 and 3, respectively). However, resistance to MTX often develops when using high-dose MTX combined with leucovorin rescue is administered and has become an important regimen in the treatment of a variety of cancers (Frei et al., 1980). Despite these preventive measures, MTX-induced resistance and toxicity continue to occur, although frequently. Mechanisms including elevated DHFR, decreased thymidylate synthase, impaired folate/antifolate transportation, and decreased polyglutamylation on MTX have been proposed to contribute to the development of MTX resistance (Asai et al., 2003). It is also postulated that the excessive use of leucovorin rescue makes tumor cells refractory to subsequent MTX therapy (Bleyer, 1977; Sirotnak et al., 1978).

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To overcome this obstacle and prevent MTX-associated toxicity, high-dose MTX combined with leucovorin rescue is administered and has become an important regimen in the treatment of a variety of cancers (Frei et al., 1980). Despite these preventive measures, MTX-induced resistance and toxicity continue to occur, although frequently. Mechanisms including elevated DHFR, decreased thymidylate synthase, impaired folate/antifolate transportation, and decreased polyglutamylation on MTX have been proposed to contribute to the development of MTX resistance (Asai et al., 2003). It is also postulated that the excessive use of leucovorin rescue makes tumor cells refractory to subsequent MTX therapy (Bleyer, 1977; Sirotnak et al., 1978). Nevertheless, what causes the emergence of MTX-resistant tumor cells and the reason why high concentrations of leucovorin might affect cell survival or even support MTX resistance remain to be answered.

In vivo, N5-CHO-THF, also known as leucovorin, is generated by the irreversible hydrolysis of N5,N10-CH2-THF in a second reaction.
catalyzed by serine hydroxymethyltransferase (SHMT). $N^5$-CHO-THF also acts as a tight-binding inhibitor of the cytosolic form of SHMT (Stover and Schirch, 1991). SHMT is a pyridoxal-5'-phosphate (PLP)-dependent enzyme and reversibly converts serine and THF to glycine and $N^5,N^{10}$-CH$_2$-THF, the principal pathway of one-carbon unit incorporation in cells. One-carbon metabolism is compartmentalized with folate coenzymes equally distributed in cytosol and mitochondria (Appling, 1991). In higher organisms more than one SHMT isoform is often present: a cytosolic isoform and an organelle-associated form, usually mitochondria. Although the physiological functions of these two isoforms remain unclear, it is suggested that cytosolic SHMT (cSHMT) tends to function in the direction of serine synthesis, whereas the production of glycine and $N^5,N^{10}$-CH$_2$-THF is mainly catalyzed by mitochondrial SHMT (mSHMT) (Narkewicz et al., 1996). Studies suggested that the activity of cSHMT is associated with the development of MTX resistance. It is believed that the cSHMT activity in vivo is modulated by the binding of $N^5$-CHO-THF and functions as a metabolic switch that shuttles the one-carbon unit between dTMP biosynthesis and homocysteine remethylation (Herbig et al., 2002). Yet the effect of $N^5$-CHO-THF on mSHMT activity and the importance of mSHMT in leucovorin metabolism and MTX resistance remain undetermined, even though the uptake of $N^5$-CHO-THF by mitochondria has been shown to be rapid and concentration-dependent (Horne et al., 1992). Currently, the animal model used for folate-related studies is restricted mostly to rodents for its resemblance with human in folate-requiring enzymes. However, deciphering the role of folate enzymes in early mammalian development might be limited because of the maternal contribution of folate coenzymes during embryogenesis (Marasas et al., 2004). Considering the feature of external development, zebrafish might serve as a valuable alternative for folate-related studies since the maternal supply of folates and folate enzymes is likely to be depleted with time in developing embryos. Increasing studies also demonstrate comparable features between zebrafish and human in many biological pathways and pathogenesis, including organ development, angiogenesis, hemostasis, heart function and circulation, apoptosis and proliferation, carcinogenesis, drug abuse and addiction, and toxicology and teratogenicity (Kari et al., 2007). However, folate-requiring one-carbon metabolism in zebrafish remains unexplored territory, albeit its critical role in oncogenesis and fetal development in vertebrates.

Our previous study on the recombinant zebrafish cSHMT revealed strong similarities with mammalian orthologs, suggesting the appropriateness of using zebrafish as a model for folate-related studies (Chang et al., 2006). In the present report, we clone and characterize zebrafish mitochondrial SHMT (zmSHMT), the other isoform of SHMT, which is less understood in mammals. To our knowledge, this is the first report on a mitochondrial folate enzyme from zebrafish that is successfully expressed and purified in Escherichia coli. The similarities revealed between zebrafish mSHMT and human orthologs add more confidence to the uses of zebrafish in folate-related studies and drug discovery. In addition, we observe differential inhibition mediated by MTX and leucovorin on the catalytic activity of these two isoforms. The potential contribution of this observation to the development of MTX resistance is also discussed.

Materials and Methods

Materials. Polymerase chain reaction (PCR) primers were ordered from MDBio, Inc. (Taipei, Taiwan). The SMART RACE Amplification Kit was purchased from Clontech, Inc. (Mountain View, CA). PCR Master Mix was purchased from ABgene House (Epsom, Surrey, UK). Restriction enzymes used for cloning procedures were purchased from either Invitrogen (Carlsbad, CA) or New England BioLabs (Ipswich, MA). The clone expressing rabbit

FIG. 1. The folate-mediated one-carbon metabolism involving both cytosolic and mitochondrial serine hydroxymethyltransferases. Folate coenzymes are equally distributed in cytosolic and mitochondrial compartments. Three cycles are involved in this pathway and are responsible for thymidylate (A), purine (B), and methionine biosynthesis (C). The enzymes participating in this pathway are serine hydroxymethyltransferase (1), dihydrofolate reductase (2), thymidylate synthase (3), glycaminide ribonucleotide transformylase (4), and 5-amino-4-imidazolecarboxamide ribotide transformylase (AICAR) (4), $N^5,N^{10}$-methyltetrahydrofolate cyclohydrolase (5), $N^5,N^{10}$-methyltetrahydrofolate dehydrogenase (6), glycaminide transformylase (7), and methionine synthase (8). A parallel pathway also exists in mitochondria, where the activated one-carbon unit is generated in the serine-aldol cleavage catalyzed by mitochondrial serine hydroxymethyltransferase.
N^5,N^10-methylenetetrahydrofolate dehydrogenase was a generous gift from Dr. Verne Schirch (Virginia Commonwealth University, Richmond, VA). The HPLC gel filtration column Alltech ProSphere SEC, 250 HR, S-200 (4.6 mm × 30.0 cm) was purchased from Alltech Associates (Deerfield, IL). (6S)-Tetrahydrofolate monoglutamate and (6S)-N^5-CHO-tetrahydrofolate monoglutamate were generous gifts from Dr. R. Moser (Merck Eprova AG, Schaffhausen, Switzerland). MitoTracker Red probes for confocal microscopy were purchased from Invitrogen. Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore Corporation (Billerica, MA). Both the Bradford assay reagent and BCA protein assay kit were purchased from Pierce (Rockford, IL). Rabbit polyclonal anti-zcSHMT antibodies were produced by Genesis Biotech Inc. (Hsinetain, Taiwan), with the enzymes we provided. Goat anti-hmSHMT antibodies, horseradish peroxidase-conjugated goat anti-rabbit IgG, and donkey anti-goat secondary antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and antibodies, buffers, amino acids, and antibiotics, were purchased from Sigma-Aldrich (St. Louis, MO).

Fish Care and Preparation of cDNA Library from Zebrafish Embryos. Zebrafish (Danio rerio, AB strain) were bred and maintained in a 14 h/10 h light-dark diurnal cycle according to the standard condition described by Westerfield (2000). Embryos were staged according to the method of Kimmel et al. (1995). Total RNA isolation and cDNA library construction from zebrafish embryos were performed with RNAzol B reagent (Tel-Test Inc., Friendswood, TX) and the SMART RACE cDNA Amplification Kit (Clontech, Inc.) as described previously (Chang et al., 2006).

Bacterial Strains, Plasmids, and General Cloning Procedures. The E. coli strain XL1 Blue (recA1, endA1, gyrA96, thi-1, hsdR17(rK^- mK^-)) (supE44, relA1, lac^-) was used for the construction of clones. The E. coli strains HMS174(DE3) (F^- recA rK^- mK^-) and Rosetta (DE3) (F^- recA rK^- mK^-), which contain the T7 RNA polymerase gene, were used for protein expression. The pET43.1a plasmid and all the E. coli strains for cloning and expression were obtained from Novagen (Madison, WI). The materials and methods for the general cloning procedures were described previously (Chang et al., 2006).

Cloning of zmSHMT from Zebrafish cDNA Library by PCR-Based Cloning Strategy. A PCR-based approach with degenerate primers was used for the amplification and cloning of SHMT-encoding sequences from a zebrafish cDNA mixture. Two degenerate primers (5'-TGGGGGNTNAAYGT-NCA-3' and 5'-WDATRTNGCCATRTC-3'), corresponding to the conserved regions of SHMT amino acid sequences (WGVNQV and DMAHIS), were designed for PCR with the following conditions: a denaturation of 94°C for 5 min followed by 55 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s. The resultant products were cloned and sequenced. The deduced amino acid sequences of the amplified products fell into two categories and shared 70 to 90% identity with the corresponding regions of SHMT. GenBank BLAST search revealed 100% and 61% identity between these two sequences and zcSHMT cDNA (zebrafish shmt1, accession number NM_201046). Based on the sequence information of the 61% identity fragment, we proceeded with the isolation of prospective zebrafish mitochondrial SHMT cDNA.

The cloning of full-length zmSHMT cDNA was accomplished by the rapid amplification of cDNA ends (RACE) method using zmSHMT gene-specific primers designed on the basis of the sequence information of the cloned fragment (Scheme 1). The reverse primer SHMT II-1(R) (5'-AGCGTGATGAGTTTGGGTCTGACATTACG-3') and the UPM primer provided in the SMART RACE cDNA Amplification Kit (Clontech, Inc.) were used in the first-round PCR, with the 5' RACE cDNA mixture as template. The resultant bands were TA-cloned and sequenced. Based on the sequence information, two primers, SHMT II-2(F) (5'-AGAGTGACTCGGGGCTGTCATTTA-3') and SHMT II-3(F) (5'-TGCTGACACTGACATTCGACAAAC-3') were designed for subsequent PCR amplifications. The SMART 3'-RACE cDNA mixture was used as template in the second-round PCR with primer pairs SHMT II-2(F) and UPM. The third round of PCR was conducted using the second-round PCR product as template and the primer pairs SHMT II-3(F) and NUP provided in the kit. All of the above amplifications were performed by touchdown PCR. The cycling conditions were 5 cycles of 94°C for 30 s and 72°C for 3 min; 5 cycles of 94°C for 30 s, 70°C for 30 s, and 72°C for 3 min; 40 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 3 min. The resulting 2700-bp fragment was identified by restriction mapping and sequencing. The assembling of 5'-RACE and 3'-RACE sequences revealed the prospective full-length zmSHMT cDNA. The final amplification of the complete 1.5-kb encoding sequence was accomplished by PCR with the 5'-RACE cDNA library prepared from 3-day post-fertilization embryos as template and the primer pair SHMT II-4(F), 5'-CCCGATGCATATGCTGACACTGACATTACG-3' (forward) and SHMT II-4(R), 5'-CCCGATGCATATGCTGACACTGACATTACG-3' (reverse). To simplify the cloning procedure, two restriction enzyme sites, NdeI and EcoRI (underlined), were introduced into the primers. The PCR-amplified product was cloned into the expression vector pET43.1a.
Successful cloning was confirmed by both restriction enzyme digestion and DNA sequencing.

Removal of signal peptide and introduction of the first methionine residue was accomplished by PCR using zmSHMT coding sequence as template with the primer pair 5'-GGCGTCTCCATGGAATGCTGGAAGC-3' (forward) and SHMT II-4 (reverse) followed by cloning into the pET34a.1 vector, yielding the clone zmSHMT(DELSig). The resultant constructs were transformed into an E. coli host cell Rosetta (DE3) for enzyme expression and purification.

Zebrafish SHMT-EGFP fusion plasmids were constructed by PCR cloning with the zcSHMT or zmSHMT plasmids and the primers designed to abolish stop codons and introduce BglII, EcoRI, or SalI restriction enzyme sites. The primers were 5'-GGCGTGGGAGATCTCCCATG-3' (forward) and 5'-CAAACAGAAGAATTGCTGTTCTGAACAC-3' (reverse) for zmSHMT/EGFP-N1; 5'-GGCGTGGGAGATCTCCCATG-3' (forward) and 5'-CCGGGGAGGTGCTGCTGGAATCC-3' (reverse) for zmSHMT/EGFP-N1.

Expression and Purification of Recombinant zmSHMT. All buffers described below for the purification of zSHMTs and kinetic studies contained 5 mM 2-mercaptoethanol, 0.2 mM EDTA, and 2 μM PLP unless otherwise stated. Similar purification procedures for zcSHMT were applied to the purification of zmSHMT(DELSig) with minor modifications indicated below (Chang et al., 2006). In brief, E. coli containing the desired plasmid was grown to log phase and induced with 0.08 mM IPTG for 3 h. Cells were harvested and lysed by lysozyme and chromatin was removed by proteamine sulfate precipitation. After a 30% to 50% ammonium sulfate fractionation, the enzyme was loaded onto a CM-Sephadex column (2.5 x 50 cm) and eluted with the linear salt gradient of 50 ml of equilibrating buffer and 50 ml of 500 mM potassium phosphate, pH 7.25. The purified enzyme was stored at −20°C or −80°C in the presence of 10% glycerol. Protein from each step of the purification was examined by SDS-PAGE for purity.

Determination of Physical Properties. Apo-SHMTs were prepared by the removal of thiazolinedione formed by l-Cys and active site PLP. The same principle was used to determine the stoichiometry of PLP bound per molecule of enzyme with an extinction coefficient of 5580 M$^{-1}$ cm$^{-1}$ for thiazolinedione (Ulevitch and Kallen, 1977). The quaternary structure was determined on a Superdex 200 size-exclusion column as described previously (Chang et al., 2006). In brief, zmSHMT was isolated, stored in 50 to 200 μl of phosphate-buffered saline, pH 7.2, and kept on ice during the whole process of extraction. Homogenization was carried out in the phosphate-buffered saline lysis buffer containing a protease inhibitor cocktail consisting of AEBSP (aprotinin, leupeptin, bestatin, pepstatin A, and E-64) (Sigma-Aldrich, product number P8340) and RNase inhibitor (Recombinant RNasin; Promega, Madison, WI). Homogenized samples were centrifuged at 10,000 g at 4°C for 10 min to remove particulate matter. Aliquots of the supernatant, about 10 to 30 μl, were subjected to Western blot and RT-PCR.

Western Blot Analysis. Supernatant protein content was determined using the Bradford (1976) and BCA methods. Proteins of 20 μg were separated on a 10% SDS-separating gel and transferred to a PVDF membrane (Millipore). After blocking in in blocking solution containing 5% nonfat milk, 0.1% Tween 20 in phosphate-buffered saline overnight, the membrane was probed with anti-zcSHMT or anti-hmSHMT primary antibodies (1:1000–1:5000) and then horseradish peroxidase-conjugated secondary antibody (1:5000). The PVDF membranes were also probed with anti-actin antibody for a loading control. The membrane was visualized using the SuperSignal chemiluminescent horseradish peroxidase substrate system from Pierce on a FUJI FILM LAS-3000 imaging system (Fuji Film, Tokyo, Japan). In the case of the gastrointestinal tract, where the signal for actin was not detectable, Ponceau-S staining was used to verify equal loading.

We used the antibody against human mSHMT instead of zebrafish mSHMT to determine zmSHMT tissue distribution, owing to the concern of possible cross-reaction between zmSHMT and zcSHMT. The human mSHMT peptide sequence is 59% and 76% identical to zmSHMT and zcSHMT, respectively. The identity between zmSHMT and zcSHMT is 61%. Thus, we hoped that the zmSHMT antibody would clearly distinguish zmSHMT from zcSHMT. As expected, no cross-reaction was detected, even when we tested with 1 μg of purified proteins, allowing the uses of the antibodies as described.

RT-PCR Analysis. For RT-PCR determination of SHMT expression, total mRNA was isolated from tissues using a TRIzol kit (Invitrogen), following the manufacturer’s instructions. After isolation, 1 μg of total mRNA in each tissue sample was reverse-transcribed with a high-capacity cDNA archive kit (Promega), and 1 μl of the newly synthesized first-strand cDNA library was used as template in the subsequent PCR analysis. The primer sequences are as follows: 5'-GAGGAGGTCGTTGTTAACAGGC-3'(F) and 5'-CATTGGAGGCGATCCCACCT-3'(R) for zmSHMT (505-bp fragment), 5'-GGGAGAAGTCGCACTCTCC-3'(R) and 5'-GGCGAGGAAAAAGCAAGGG-3'(R) for zmSHMT (523-bp fragment), 5'-AGACATGCAAGGAGAAACG-3'(F) and 5'-TCCAGACGAGATTTAC3'(R) for β-actin (391-bp fragment) as a control for the RNA isolation and reverse-transcription. The annealing temperatures were 65°C for zcSHMT, 60°C for zmSHMT, and 62°C for β-actin. The PCR condition was 30 cycles of 30 s at 94°C, 30 s at annealing temperature, and 68°C for 30 s.

Determination of Intracellular Localization. ZLE cells were cultivated and regularly maintained in Leibovitz’s L-15 medium supplemented with 5% fetal bovine serum at 28°C. For transient transfection, ZLE cells at 1 × 105/ml were subcultured into six-well plates 24 h before transfection with zmSHMTs/ pEGFP-N1 fusion plasmids with a si-PORT transfection kit (Ambion, Austin, TX). Cells were incubated for another 24 h and costained with mitochondrial probe MitoTracker Deep Red 633 (Invitrogen) right before examining under a confocal microscope. Confocal microscopy images were acquired on a Leica TCS SP2 microscope.

Results

Cloning and Sequence Analysis of Recombinant zmSHMT. The sequences of the 370-base pair fragments resulting from PCR amplification with degenerate primers fall into two categories, designated as form I and form II. We had previously reported the cloning and characterization of zmSHMT form I, the prospective zmSHMT, which highly resembles mammalian cytosolic SHMT structurally and functionally (Chang et al., 2006). The full-length zmSHMT form II cDNA isolated is 1479 bp, which encodes a protein of 492 amino acids. Peptide sequence alignment with the known SHMT from other species and the prospective zmSHMT revealed a potential mitochondrial signal peptide cleavage site between residues 20 and 30. Signal peptide prediction using the software SignalP 3.0 (http://www.cbs. dtu.dk/services/) has narrowed the cleavage site down to Ala22 and Val24, and is in agreement with the reports for rabbit and human
mitochondrial SHMTs (Fig. 2) (Martini et al., 1989; Stover et al., 1997). This prospective mitochondrial leader sequence of zSHMT form II (which we now designate as zmSHMT) is rich in arginine, leucine, threonine, and serine, and fulfills the criteria for a mitochondrial targeting sequence that locates at the N terminus of the precursor protein and contains 17 to 35 amino acids rich in positive charge. Both the conserved octapeptide encompassing a stretch of threonine residues and the lysine residue forming the internal aldimine with PLP are also identified in zmSHMT, further supporting the notion that zebrafish SHMTs are the orthologs of mammalian SHMTs (Fig. 2). The peptide sequence of zmSHMT is 76% identical to its human ortholog, indicating a high conservation throughout evolution.

Expression and Purification of zSHMTs. The overexpressed full-length zmSHMT with leader peptide resulted in the formation of insoluble precipitate in E. coli at all the growth conditions we had tested, including lower temperatures, reduced inducer concentrations, and addition of cofactor PLP and low molecular weight glycols (data not shown). The attempt to obtain a soluble and fully functional protein succeeded only after we performed a second PCR-based cloning to eliminate the predicted leader peptide that includes the first 23 amino acids. Induction for zmSHMT without signal peptide, designated as zmSHMT(DelSig), reaches an acceptable level with minimal production of insoluble enzyme (Fig. 3). Higher concentrations of IPTG, elevated induction temperature, and/or prolonged induction time were found to increase the ratio of insoluble to soluble zmSHMT(DelSig), although the overall amount of induced enzyme is increased.

A purification protocol similar to those for zcSHMT and human SHMTs was applied to the purification of zmSHMT(DelSig) (Kruschwitz et al., 1995; Chang et al., 2006). After a 30 to 50% ammonium sulfate precipitation, the enzyme was 70% pure, judged from SDS-PAGE, with a 2-fold purification (Table 1). We used gel filtration, instead of equilibrium dialysis, to remove ammonium sulfate, because zmSHMT(DelSig) was found to precipitate after overnight dialysis. The zmSHMT(DelSig) bound to CM-Sephadex and was eluted at high salt. Chromatographing on CM-Sephadex separates the endogenous enzyme from our cloned zmSHMT, since most E. coli proteins, including E. coli SHMT, do not bind to cation exchange resins under conditions of high ionic strength.
these conditions (di Salvo et al., 1998). Therefore, this step greatly simplifies the purification procedure and permits removal of the great bulk of unwanted protein, with a large -fold of purification achieved. The SHMT eluted from this column was better than 98% pure (Fig. 3). From 2 liters of cells, approximately 43 mg of pure zmSHMT(DelSig) was obtained with an overall yield of 90% (Table 1). The purified recombinant enzyme can be stored at −20°C or −80°C in the presence of 10% glycerol for at least 6 months without significant change in catalytic activity. However, frequent freeze-thaw cycles result in protein precipitation and loss of enzymatic activity.

**Physical Properties of zmSHMT(DelSig).** The spectral properties of the recombinant zmSHMT(DelSig) seem to be similar to those of most of the studied SHMTs, including human (Kruschwitz et al., 1995; di Salvo et al., 1998; Chang et al., 2006). Beside 278 nm, zmSHMT (DelSig) displays a distinct absorbance peak at 428 nm, corresponding to the internal aldimine formed between PLP and an active site lysine residue (Fig. 4). The 428-nm peak in all other studied SHMTs gives a distinct spectral change in the presence of glycine and reduced folates due to the formation of a quinonoid ternary complex absorbing near 500 nm. This long wavelength absorbance is attributed to a glycine anion in resonance with the bound pyridoxal phosphate and has been used extensively to determine the binding constants of tetrahydrofolates and glycine (Schirch, 1982). The same properties were observed for the recombinant zmSHMT(DelSig) (Fig. 4).

The predicted molar absorptivity coefficient at 278 nm for denatured zmSHMT(DelSig) was 39,910 M⁻¹ cm⁻¹, based on amino acid composition. The coefficient of the native enzyme was obtained by multiplying the predicted value by the ratio of absorbance at 278 nm for denatured and native states, yielding a molar absorptivity coefficient of zmSHMT(DelSig) ε₂₇₈ = 45,893 M⁻¹ cm⁻¹. This shows that a 1-mg/ml solution of zmSHMT(DelSig) holoenzyme will exhibit absorption of 0.88 at 278 nm. This level is slightly higher, but a 1-mg/ml solution of zmSHMT(DelSig) monomer will exhibit a molar absorptivity coefficient of 45,960 M⁻¹ cm⁻¹ (Chang et al., 2006). The Trp residues contained in zmSHMT and zmSHMT are two and three, respectively.

SDS-PAGE showed a single band of approximately 50 kDa for the recombinant zmSHMT(DelSig) (Fig. 3). This compares to the calculated size of 51,912 Da based on the peptide sequence of a zmSHMT(DelSig) monomer. Both holo- and apo-zmSHMT(DelSig) had a Stokes radius close to a globular protein of 200 kDa and were eluted at the same retention volume as zmSHMT and human cytosolic SHMT tetramers on a Superdex 200 column (data not shown). These results suggest a homotetrameric structure for the recombinant zmSHMT(DelSig).

1-Cysteine forms a thiazolidine compound with the active site PLP, which can be removed by dialysis or precipitation of the protein, providing a simple method for preparing mitochondrial apo-SHMT and determination of PLP binding stoichiometry (Ulevitch and Kallen, 1977). Our results show that one PLP molecule binds to each zmSHMT(DelSig) monomer, as observed for zcSHMT and most of the SHMTs studied to date (data not shown).

**Steady-State Kinetic Constants and Reduced Folate Affinity.** Double-reciprocal plots of initial velocity versus serine concentration permit the determination of both apparent Km for serine and kcat. As shown in Table 2, both the Km of serine and the kcat of zmSHMT(DelSig) are comparable to the values for zcSHMT and rabbit mitochondrial SHMT. The enzyme remains fully active at 37°C for at least 30 min (data not shown).

The quinonoid intermediate formed between reduced folates and the active site PLP absorbs near 500 nm with a molar extinction coefficient of 40,000 M⁻¹ cm⁻¹ (Schirch et al., 1977). This absorbance shows saturation kinetics with most reduced folate substrates, including H4PteGluα, N5-CHO-H4PteGluα, and N5-CHO-H4PteGluβ (Schirch and Ropp, 1967; Stover and Schirch, 1991). The binding of substrates to rabbit cytosolic SHMT is a sequential random mechanism. Previous studies had confirmed that the Kd values determined by this method were essentially the same as the Km values (Kruschwitz et al., 1977; Szebenyi et al., 2004). However, the reported value is an apparent Kd because the formation of this complex is at least a two-step process. The lower Kd of zmSHMT(DelSig) for THF suggests a higher affinity for this substrate compared with zcSHMT. Both isoforms have comparable affinity for N5-CHO-THF, as judged from their similar dissociation constants (Table 2).

**Inhibition of SHMT Aldol Cleavage Activity.** Increasing concentrations of leucovorin (N5-CHO-THF) inhibit both zcSHMT and hcSHMT activities substantially, yet to a lesser extent than zmSHMT (Fig. 5). The inhibitions of SHMT-catalyzed serine aldol cleavage by leucovorin and MTX were determined for zc-, zm-, and hcSHMT. Approximately 70% and 30% inhibition were observed for zc- and zmSHMT(DelSig) activities, respectively, in the presence of 70 μM N5-CHO-THF (Fig. 5A). The IC50 of leucovorin is approximately 30 μM for zmSHMT and higher than 70 μM for zcSHMT. The differential inhibition is evident with the presence of 10 μM leucovorin, the concentration estimated in serum in a high-dose leucovorin rescue regimen. A similar pattern of inhibition, but an even larger difference between zc- and zmSHMT was observed when the inhibition was assayed in the presence of 50 μM serine (Fig. 5B). MTX also represses SHMT activities, but not as significantly as it does zebrafish DHFR activity (Fig. 5C; T. F. Fu, unpublished result). No significant difference was observed when the highest concentrations of leucovorin (70 μM) and MTX (100 μM) were added simultaneously to the reaction in a combined assay compared with adding leucovorin alone (data not shown).

**Tissue-Specific Distribution of zmSHMT and zcSHMT Isoforms.** RT-PCR results showed that zmSHMT mRNA was evenly distributed among tissues, whereas significantly higher levels of zcSHMT mRNA were detected in heart, liver, and ova (Fig. 6C). This result is in agreement with the ubiquitous distribution of human mSHMT message and tissue-specific expression of cSHMT mRNA (Girgis et al., 1998). PCR was also performed using plasmids containing zcSHMT or zmSHMT coding sequences as templates to
validate the positive signal. No PCR product was observed in this cross-reaction test (Fig. 6A).

Interestingly, we found that the SHMT protein levels did not correspond to their mRNA amounts detected in most of the tissues examined. Strong tissue specificity was observed in protein levels for both zcSHMT and zmSHMT (Fig. 6C). Zebrafish cSHMT protein was predominant in liver and also abundant in ova. Significant zmSHMT protein was detected only in liver and gastrointestinal tract regardless of the evenly distributed mRNA message. We also noted that appreciable amounts of cSHMT, but not mSHMT, were found in unfertilized eggs in both mRNA and protein levels. Equal loading of samples was confirmed by the presence of β-actin, Ponceau-S staining of the membrane, and Coomassie Brilliant Blue-stained SDS-polyacrylamide gel.

**Subcellular Localization of Zebrafish SHMTs.** The prediction on the recombinant zSHMT’s intracellular localization was confirmed by the site-specific compartmentalization of EGFP-fused SHMTs with confocal microscopy. The overexpressed zmSHMT-EGFP was clearly colocalized with a mitochondrial marker, demonstrating the mitochondrial localization of this enzyme (Fig. 7A). For zcSHMT-EGFP, surprisingly, the fluorescence signal of various intensities was detected in both nucleus and cytosol. No signal corresponding to free GFP was detected in cell extracts prepared from zcSHMT-EGFP transformants, excluding the possibility of artifacts or false signal due to any undesired sample contamination (Fig. 7B).

**Discussion**

Despite the unknown biological function and significance of SHMTs, studies showed that impairment of SHMT activity resulted in disturbance of homeostasis of the intracellular one-carbon pool and led to pathogeneses including homocysteinemia, cancers, cardiovascular diseases, and neural tube defects, implying a crucial role of SHMT in normal cell growth and functions (Stover and Garza, 2006). The existence of both cytosolic and mitochondrial SHMTs has been known since Nakano et al. (1968) partially purified the enzyme from rat liver mitochondria in 1968. Since then, cytosolic SHMTs of many species have been studied thoroughly, whereas information about the mitochondrial isoform is still very limited.

We report here the cloning and characterization of zebrafish mitochondrial SHMT. The identity of this recombinant protein was confirmed by its serine-aldol cleavage activity and colocalization with MitoTracker Red, a mitochondrial specific dye. The full-length zm-SHMT expressed in *E. coli* forms inclusion bodies. This result was not unexpected since expression of organelle-specific proteins containing signal peptide often leads to formation of insoluble protein. Further characterization of zmSHMT(DelSig) reveals substantial similarities in its structure, physical properties, and kinetics to zcSHMT and mammalian orthologs, adding confidence to using zebrafish as an animal model for folate-related studies. That similar protocols applied to the purification of hcSHMT, rcSHMT, zcSHMT, and zmSHMT(DelSig) suggests comparable surface properties among these isoforms. Surprisingly, no evident cross-reaction between anti-zcSHMT antibody and zmSHMT(DelSig) protein was detected in Western blot analysis, despite a 63% identity being found in their peptide sequences. The possible explanation is that the homologous sequences might be embedded inside and therefore were not exposed to lymphocyte recognition and antibody generation.

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<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Total Activity (U)</th>
<th>Specific Activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lysate</td>
<td>19</td>
<td>537</td>
<td>736</td>
<td>1.37</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>((\text{NH}_4)_2\text{SO}_4)</td>
<td>15</td>
<td>297</td>
<td>825</td>
<td>2.77</td>
<td>120</td>
<td>2</td>
</tr>
<tr>
<td>CM</td>
<td>2</td>
<td>43</td>
<td>658</td>
<td>15.3</td>
<td>90</td>
<td>11.2</td>
</tr>
</tbody>
</table>

**TABLE 1**

Summary of recombinant zmSHMT(DelSig) purification

**FIG. 4.** Absorbance of zebrafish SHMTs and its complexes with glycine and folate substrates. A, spectrum of zcSHMT (solid line) and zmSHMT(DelSig)(dotted line) in 20 mM potassium phosphate at pH 7.0. B, spectrum of zmSHMT(DelSig) (curve 1), zmSHMT(DelSig) saturated with glycine (curve 2), zmSHMT(DelSig) saturated with glycine and \( H_4\text{PteGlu} \) (curve 3), and zmSHMT(DelSig) saturated with glycine and \( N^5\)-CHO-\( H_4\text{PteGlu} \) (curve 4).

validate the positive signal. No PCR product was observed in this cross-reaction test (Fig. 6A). Interestingly, we found that the SHMT protein levels did not correspond to their mRNA amounts detected in most of the tissues examined. Strong tissue specificity was observed in protein levels for both zcSHMT and zmSHMT (Fig. 6C). Zebrafish cSHMT protein was predominant in liver and also abundant in ova. Significant zmSHMT protein was detected only in liver and gastrointestinal tract regardless of the evenly distributed mRNA message. We also noted that appreciable amounts of cSHMT, but not mSHMT, were found in unfertilized eggs in both mRNA and protein levels. Equal loading of samples was confirmed by the presence of β-actin, Ponceau-S staining of the membrane, and Coomassie Brilliant Blue-stained SDS-polyacrylamide gel.

**Subcellular Localization of Zebrafish SHMTs.** The prediction on the recombinant zSHMT’s intracellular localization was confirmed by the site-specific compartmentalization of EGFP-fused SHMTs with confocal microscopy. The overexpressed zmSHMT-EGFP was clearly colocalized with a mitochondrial marker, demonstrating the mitochondrial localization of this enzyme (Fig. 7A). For zcSHMT-EGFP, surprisingly, the fluorescence signal of various intensities was detected in both nucleus and cytosol. No signal corresponding to free GFP was detected in cell extracts prepared from zcSHMT-EGFP transformants, excluding the possibility of artifacts or false signal due to any undesired sample contamination (Fig. 7B).

**Discussion**

Despite the unknown biological function and significance of SHMTs, studies showed that impairment of SHMT activity resulted in disturbance of homeostasis of the intracellular one-carbon pool and led to pathogeneses including homocysteinemia, cancers, cardiovascular diseases, and neural tube defects, implying a crucial role of SHMT in normal cell growth and functions (Stover and Garza, 2006). The existence of both cytosolic and mitochondrial SHMTs has been known since Nakano et al. (1968) partially purified the enzyme from rat liver mitochondria in 1968. Since then, cytosolic SHMTs of many species have been studied thoroughly, whereas information about the mitochondrial isoform is still very limited.

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pattern might also occur in vivo (Vinnars et al., 1975). The differential inhibition observed between these two isoforms might be attributed to the lower $K_d$ of zmSHMT than of zcSHMT for THF. Stover and colleagues suggested that $N^5$-CHO-THF binds and modulates cSHMT activity, enabling this enzyme to function as a regulatory switch in one-carbon metabolism. When activated, the cSHMT-derived $N^5,N^{10}$-CH$_2$-THF gives the thymidylate synthetic pathway higher metabolic priority than the homocysteine remethylation cycle (Herbig et al., 2002; Woeller et al., 2007). The latter one generates $S$-adenosyl methionine, the major methyl donor for most intracellular methylation including DNA and protein. Our results add further weight to the notion that zmSHMT is responsible for a stable supply of $N^5,N^{10}$-CH$_2$-THF, whereas cSHMT is sensitive to alteration in nutritional status and functions to regulate the one-carbon flow in a changed environment (Stover and Garza, 2006). In a high-dose MTX combined leucovorin rescue therapy, the differential inhibitory effects of $N^5$-CHO-THF to zc- and zmSHMT might result in a decreased ratio of THF to $N^5,N^{10}$-CH$_2$-THF, and hence, redistribution of the activated one-carbon units between nucleotide biosynthesis and cellular methylation, yielding profound impact in intracellular events, gene activities, and, ultimately, cell survival. Evidence supporting the notion that cSHMT activity might play a role in the development of MTX

### Table 2

Comparison of kinetic parameters for recombinant zmSHMT(DelSig) with zcSHMT and rmSHMT

<table>
<thead>
<tr>
<th>Species</th>
<th>$K_m$ for serine (mM)</th>
<th>$K_d$ for $H_4PteGlu$ (μM)</th>
<th>$K_d$ for $N^5$-CHO-$H_4PteGlu$ (μM)</th>
<th>Turnover Number (min$^{-1}$)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zebrafish m$^a$</td>
<td>0.43</td>
<td>8</td>
<td>2.8</td>
<td>537</td>
<td>Present report</td>
</tr>
<tr>
<td>Zebrafish c$^b$</td>
<td>0.22</td>
<td>18</td>
<td>2.6</td>
<td>351</td>
<td>Chang et al. (Chang et al., 2006)</td>
</tr>
<tr>
<td>Rabbit m$^c$</td>
<td>0.60</td>
<td>25</td>
<td>10</td>
<td>500</td>
<td>Schirch et al. (Schirch and Peterson, 1980)</td>
</tr>
</tbody>
</table>

$^a$ zebrafish mSHMT(DelSig); $^b$ cytosolic SHMT; $^c$ mitochondrial SHMT

### Figure 5

Inhibition of the SHMT-catalyzed serine-aldol cleavage by increasing concentrations of leucovorin ($N^5$-CHO-THF) (A and B) and methotrexate (C). Inhibition on initial velocity was determined at 30°C in the presence of 25 μM $H_4PteGlu$, saturated NADP$^+$, 10 mM (A and C) or 50 μM (B) serine, 0.1 μM SHMT, and inhibitors ranging from 0.1 to 100 μM.
resistance has been reported. In support of this notion are that polymorphism in cSHMT was related to MTX resistance in pediatric patients with acute lymphoblastic leukemia, and overexpression of cSHMT in Leishmania increased resistance to methotrexate in a rich folate-containing medium (de Jonge et al., 2005; Gagnon et al., 2006). The present study adds mSHMT to the picture for possible mechanistic insights and provides clues to further understand the complex relationships between one-carbon metabolism, SHMTs, and the development of MTX resistance.

The MTX and leucovorin concentrations used in the inhibition studies ranged from 0.1 to 100 μM. It was estimated that a concentration of 1 to 10 μM for both MTX and leucovorin is a realistic serum concentration that can be reached in a MTX-leucovorin combined regimen (Widemann and Adamson, 2006). However, we are convinced that the differential inhibition observed in our studies should have reflected what has occurred in vivo, since the polyglutamylation of folate/antifolate substrates will significantly increase their affinities to folate enzymes. Five to seven glutamate residues will be added to the γ-carboxyl group of the internalized MTX and N^5^-CHO-H_4PteGlu_5, respectively (Huang et al., 1998; Fu et al., 2005). This result implies that the inhibition mediated by N^5^-CHO-H_4PteGlu_5 in cells should be comparable to, if not more significant than, the results observed in in vitro studies since polyglutamylation will further potentiate the competitiveness of polyglutamylated leucovorin with tetrahydrofolate polyglutamate.

Tissue-specific expression was evident in the protein level for both SHMT isozymes in zebrafish, with the highest expression in liver. This is, to our knowledge, the first report on the tissue-specific distribution of mitochondrial SHMT. It was documented that human mSHMT mRNA was evenly expressed among tissues (Girgis et al., 1998). Interestingly, our RT-PCR results for zmSHMT were in agreement with the human mSHMT expression pattern and showed equal distribution, suggesting that translational and/or post-translational regulation might play a role in controlling the intracellular concentrations of both enzymes. Protein stabilized by the binding of folate substrates and/or PLP cofactor might also contribute to the different protein levels observed in this study. Support for this notion is very recent, showing that lack of vitamin B_6 in cells causes a decrease in SHMT protein but not mRNA level (Perry et al., 2007). Notably, cSHMT is abundant in unfertilized eggs, supporting the view that SHMT is a maternally essential gene (Vatcher et al., 1998). Studies on the cor-

![Fig. 6. Tissue distribution of zebrafish cytosolic and mitochondrial SHMTs. A, plasmids (20 ng) containing SHMT coding sequences were used as templates in PCRs for specificity determination with the primer pairs indicated. B, anti-zcSHMT and anti-hmSHMT antibodies were tested against purified zcSHMT and zmSHMT for specificity. One microgram of each purified protein was subjected to SDS-PAGE and analyzed by Coomassie Brilliant Blue (CBB) staining (top) and Western blotting with anti-hmSHMT (middle) and anti-zcSHMT (bottom) antibodies. C, individual tissues were prepared for Western blot (top) and RT-PCR (bottom) analysis from adult female zebrafish as described under Materials and Methods. D, tissue extracts containing 20 μg of protein were separated by 10% SDS-PAGE and visualized by Coomassie Brilliant Blue staining. Results presented here are representative of six independent repeats. GI, gastrointestinal.](https://example.com/fig6.jpg)
relation between the abundance of SHMTs and the potential risk of developing MTX resistance might be rewarded by an understanding of the differential efficacy of MTX observed in various types of cancer.

We were puzzled and vigilant when we observed the presence of zcSHMT in the nucleus, since the nuclear localization of cSHMT had never been reported at that time. A negative result was obtained when the peptide sequence was subjected to a search for a specific nuclear-targeting sequence. Having repeated this experiment carefully and revealed the same results many times prompted us to postulate that the zcSHMT-EGFP might be transported into nucleus via cargo or other component-mediated mechanisms. Interestingly, our observation and hypothesis were found later to be in agreement with what was reported by Woeller et al. (2007) in a very recent study showing that human cSHMT was SUMOylated and nuclear-localized in a cell cycle-dependent manner. Two prospective SUMOylation sites are identified in the zcSHMT peptide sequence, suggesting a possibly similar mechanism for zcSHMT nuclear localization and a resemblance between human and zebrafish SHMTs. The biological significance of the nuclear-localized cSHMT is unknown. Yang and Meier (2003) showed that cSHMT was in some way connected to the nucleolar protein SRP40p and modulated cell cycle and cell size, supporting a noncatalytic function of cSHMT. Enlargement of the cell size was also observed in our zcSHMT-EGFP-transfected cells. The

![Fig. 7. Localization of EGFP-tagged zSHMTs in ZLE cells. A, the EGFP fusion constructs with zmSHMT (top) or zcSHMT (middle and bottom) fused to the N terminus of EGFP were transiently transfected into ZLE cells, and live confocal images were taken at 24 h after transfection. The scales at the lower right corner for zmSHMT-EGFP and zcSHMT-EGFP transfectants are 8.00 μm and 20.00 μm, respectively. B, extracts containing 20 μg of protein prepared from cells transfected with plasmid generating free EGFP (lane 1) or zcSHMT-EGFP fusion protein (lane 2) were subjected to 10% SDS-PAGE and Western blot analysis with antibodies indicated on the top.](image-url)
significance of zcSHMT intracellular localization is currently under investigation. Zebrafish have attracted the interest of many researchers as an animal model in the past two decades. The features of external development, transparent embryo, ease of growth and breeding, economy, and ease of manipulation using well established molecular approaches have made zebrafish an ideal animal model for studying developmental biology and pathogenic mechanisms in a variety of conditions. Especially important for drug discovery is that zebrafish embryos are permeable to small molecules and drugs during organogenesis, providing easy access for drug administration and vital dye staining (Kari et al., 2007). There is no doubt that efforts will be continually invested in the improvement of antifolate drugs, considering the vital role of folicates in nucleotides and protein biosynthesis. The search for new targets of antifolate drugs will also be sustained. In addition to its vital role in folate-mediated one-carbon metabolism, the property of being highly expressed in rapidly proliferating cells has made SHMTs a potential target for chemotherapy and immunosuppression (Renwick et al., 1998). Our studies conclude that zebrafish SHMTs share high similarity with human isozymes, indicating that zSHMTs are more than a whole, are appropriate systems for folate-related studies and antifolate drug discovery. Further studies on other folate enzymes should be warranted. In addition, the possible mechanistic insights provided in this study enable us to further understand the complex relationships between one-carbon metabolism, SHMTs, and the development of MTX resistance.

Acknowledgments. Our sincere appreciation goes to Dr. Verne Schirch, Virginia Commonwealth University, for valuable advice and assistance. We also thank Dr. R. Moser, Merck Eprova AG, and Dr. Jian-Ruey Hong, National Cheng Kung University, for the precious materials they generously provided.

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